Non-invasive hepatic biomarkers (ELF and CK18) in people with type 2 diabetes

Non-invasive hepatic biomarkers (ELF and CK18) in people with type 2 diabetes: the Edinburgh Type 2 Diabetes Study

[Running head: ELF and CK18 in type 2 diabetes]

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Abstract

Background

Type 2 diabetes is an established risk factor for the presence and progression of fatty liver. Little is known about the distributions and correlates of hepatic non-invasive biomarkers in community based populations with diabetes, unselected for liver disease.

Aims

We aimed to identify the distribution of, and metabolic risk factors associated with serum cytokeratin-18 (CK18) and the Enhanced Liver Fibrosis score (ELF), in a large, representative cohort of people with type 2 diabetes (the Edinburgh Type 2 Diabetes Study, ET2DS).

Methods

939 ET2DS participants, aged 60-74 years underwent physical examination including ultrasound for assessment of liver fat. Representative subgroups were assessed for markers of chronic liver disease (CK18 and ELF).

Results

CK18 values ranged from 29-993 U/L (median 102, IQR 76-137 U/L) and ELF scores ranged from 6.9-11.6 (mean 8.9, SD 0.8). Statistically significant associations were found between both biomarkers and a number of metabolic risk factors. Neither CK18 nor ELF was consistently or strongly associated with established hepatic risk factors (alcohol excess, hepatotoxic medication use and positive immunology titres).

Conclusions

We identified the distribution of CK18 and ELF in a large cohort of older people with type 2 diabetes and showed that these markers are associated with an adverse metabolic risk factor profile, although much of the variation in biomarkers remained unexplained. Prospective studies are required to determine the extent to which CK18 and/or ELF predict the development of symptomatic liver disease and to identify additional risk factors which may influence the development of advanced liver disease in people with type 2 diabetes.

Keywords

Non-invasive hepatic biomarkers, cytokeratin-18, Enhanced Liver Fibrosis score, fatty liver disease, type 2 diabetes mellitus

Introduction

Type 2 diabetes is an established risk factor for fatty liver in Western countries(1). The commonest cause of fatty liver, non-alcoholic fatty liver disease (NAFLD), ranges from simple steatosis (non-alcoholic fatty liver, NAFL), through to liver inflammation (non-alcoholic steatohepatitis, NASH) and on to NASH with liver scarring (fibrosis) and cirrhosis (end stage fibrosis). The consequences of the more advanced stages of NAFLD, including cirrhosis, liver failure and hepatocellular carcinoma (2) are becoming increasingly common; NAFLD is the third most frequent indication for liver transplantation in the USA and transplants performed with NAFLD as the primary aetiology rose from 1.0% in 2001 to 8.5% in 2009(3). Even without transplant, NASH and advanced stages of liver disease are associated with higher individual healthcare costs (4). In type 2 diabetes, significant mortality related to chronic liver disease (CLD) has been reported(5) with a standardised mortality ratio (SMR) of 2.52 for liver cirrhosis compared with the general population in a European cohort (compared with a SMR for cardiovascular disease of 1.34)(6).

Given the potential burden caused by symptomatic (advanced) liver disease in the diabetic general population, there is a need to investigate potential methods of identifying asymptomatic stages of the condition in adults with diabetes, as indeed is the case for other high risk sub-groups within the general population. Liver biopsy, which to date has been the mainstay of liver fibrosis diagnosis, has limited usefulness in the investigation of large groups of people from healthy populations (i.e. those unselected for liver disease) because of its invasive nature, complication rates(7), sampling errors(8) and inter-observer variability(8). Therefore, interest is increasing in the use of non-invasive biomarkers of liver inflammation and fibrosis which might be useful in the identification of particularly high risk groups of individuals.

In the current study, we sought to determine the distribution, and factors influencing levels of, two promising non-invasive liver markers, serum cytokeratin-18 (CK18) and the Enhanced Liver Fibrosis (ELF) score. These markers have previously been validated for hepatic inflammation and fibrosis respectively against liver biopsy in patients with established chronic liver disease attending tertiary care settings. CK18, a caspase cleaved fragment released by injured hepatocytes and a measure of hepatic cell damage such as inflammation in NASH, is raised in patients with CLD compared with people without CLD (9) and can differentiate between steatosis and NASH(10-12)in patients with NAFLD (14-16). ELF uses an extra-cellular matrix panel (hyaluronic acid (HA), N-terminal pro-peptide of collagen type III (P3NP) and tissue inhibitor of metalloproteinase-1 (TIMP-1) to quantify fibrosis and has been validated for use in patients with NAFLD(13) with increasing accuracy for severe fibrosis detection compared to earlier stages. However, neither biomarker has been validated in general population-based cohorts, nor in diabetic populations, an important issue which has been hampered to date by lack of information on their distribution and clinical correlates in such populations.

A recent study in South Korea reported on the normal distribution of ELF in adults without known CLD(14). This unique study of a large group of patients with type 2 diabetes, unselected for liver disease; investigates the distribution and clinical correlates of non-invasive markers of liver fibrosis and inflammation. Adding to previous studies which have focussed on hospital outpatient settings and the use of liver biopsy(15, 16). Such information is necessary for the purpose of screening and treatment of undiagnosed liver disease in diabetic patients and also to underpin further research into the causes and consequences of asymptomatic liver disease in adults with diabetes.

Patients and Methods

The Edinburgh Type 2 Diabetes Study

Recruitment and examination of ET2DS subjects have been published elsewhere(17). In brief, 1066 men and women aged 60 to 75 years were recruited at random from the Lothian Diabetes Register. ET2DS participants have been shown previously to be representative of all those randomly selected to participate in the study (n=5454), and of the target population of older people with type 2 diabetes living in the general population(18). One year after recruitment and baseline examination, 939 participants (88%) returned for further clinical and liver assessment (19, 20). Subjects returning at year 1 were similar to the full ET2DS population in terms of a number of variables including demographics, body fat measures, glucose and HbA1c measures, lipid profiles, blood pressure and medication use(18, 19).

Clinical examination

Clinical examination included a fasting blood sample for measurement of plasma glucose, HbA1c, total cholesterol, triglycerides, estimated glomerular filtration rate (eGFR), alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyltransferase and platelets; measurement of height, weight, and waist circumferences; a self-administered questionnaire including questions on year of diabetes diagnosis, current medications, alcohol consumption, history of joint and liver disease. In addition, patients underwent abdominal ultrasound scan (USS) and those participants with evidence of hepatic steatosis or plasma liver enzymes above the laboratory reference limits received a 'liver screen' including: hepatitis B virus serology, hepatitis C virus serology, alfa-fetoprotein, ferritin and autoantibodies (anti-nuclear antibody (ANA), anti-mitochondrial antibody (AMA) and anti-smooth muscle antibody (ASmA))(19). CK18 and ELF were measured in stored serum samples (-80C), taken at the time of the liver USS.

Average alcohol intake per week over the previous year and history of alcohol excess were determined from two questions in the self-completion questionnaire, adapted from the AUDIT-C screening tool(21): "How often did you have a drink containing alcohol in the past year?"(a drink was considered to be one and a half alcohol units); and "How many drinks did you have on a typical day when you were drinking in the last year?". Self-reported data on potential hepatotoxic medication use within the previous 6 months were confirmed by review of medical records.

Identifying pre-diagnosed liver disease

The presence of liver disease diagnosed prior to attendance at the research clinic was identified from data linkage to SMR01 general and acute inpatient discharge records (at NHS National Services Scotland, Information Services Division) and from questions on prior health condition in the patient questionnaires. Diagnoses were verified by review of medical records. Patients with confirmed chronic viral hepatitis, haemochromatosis and primary biliary cirrhosis were excluded from the final analyses as biomarkers are known to perform differently in these conditions.

Defining hepatic steatosis

Hepatic steatosis was determined by abdominal USS as described previously(22). The same sonographer, blinded to the participants' clinical history, undertook all scanning and grading. The liver was graded for markers of hepatic steatosis using established criteria: bright hepatic echo pattern (when compared to the right kidney), increased attenuation of the echo beam (visualised as poor imaging of the diaphragm or intrahepatic vessels) and the presence of focal fatty sparing.(19, 23-25). In a subset, sonographic steatosis was validated using magnetic resonance spectroscopy with an optimal fat fraction cut-off of 6%(22) following

which grades were defined as: normal (fat fraction <6.1%) or significant steatosis (fat fraction >6%). Radiological signs of cirrhosis were also noted and spleen size was measured in cm.

Laboratory Measurements

CK18 was measured using the M30-Apoptosense® ELISA (PEVIVA AB, Stockholm, Sweden) at the Biomedical Research Unit laboratory (University of Nottingham, UK) and ELF using the ADVIA Centaur immunoassay system (Siemens Healthcare Diagnostics Inc, New York, USA) at the iQur laboratory (London, UK). The individual ELF markers are combined using the algorithm ELF=2.588+(ln(HA)*0.681)+(ln(P3NP)*0.775)+(ln(TIMP1)*0.494)(13). All other biochemical variables were analysed using a Vitros Fusion chemistry system (Ortho Clinical Diagnostics, Bucks, UK) at the Western General Hospital (Edinburgh, UK). AST/ALT ratio was calculated as AST(U/L)/ALT(U/L) and the aspartate to platelet ratio index (APRI) was calculated as [[AST(U/L)/upper limit normal]/platelets($x10^9$/L)]*100.

Data Analysis

Data were analysed using SPSS v19.0 (SPSS Inc., Illinois, USA). Alcohol excess was defined according to established criteria as alcohol intake >14 units/week (female) or >21 units/week (male)(26), or participant self-report of current/previous alcohol excess(2). Use of hepatotoxic medication included the use of (non-topical) glucocorticoids for >2 weeks, isoniazid, methotrexate, amiodarone, or tamoxifen within the 6 months prior to USS(2, 27). Clinically significant positive immunology titres were defined as ASmA titre >1:160 or AMA titre >1:40(2, 28). CKD was defined as eGFR<60 mls/min/$1.73m^2$. Patients were considered to have arthritis if they reported a history of osteoarthritis, rheumatoid arthritis, scleroderma or any other joint disease. Continuous variables were assessed for normality with CK18, duration of diabetes and triglycerides requiring transformation for analysis.

The associations of CK18 and ELF with the following were examined, (i) duration of diabetes and diabetes treatment categorised as diet-controlled, oral anti-hypoglycaemic agents (OAHA) only or insulin +/- OAHA,), (ii) metabolic variables (total cholesterol, triglycerides, fasting glucose, HbA1c, BMI calculated as weight (kg)/height $(m)^2$ and waist circumference), (iii) steatosis on USS and, (iv) established risk factors for CLD (alcohol excess, hepatotoxic medication, positive immunology). The influence of CKD and arthritis (on circulating biomarker level) was assessed by analysing their prevalence in the highest and lowest quintiles of each biomarker. Analyses were undertaken on (i) all subjects, (ii) subjects with steatosis (defined as the presence of steatosis on USS) and, (iii) subjects with NAFL (defined as the presence of steatosis on ultrasound without alcohol excess, use of hepatotoxic medication or raised autoantibodies).

Univariate analysis of potential risk factors was undertaken using Pearson's correlation and ANOVA adjusting for age and sex. Multivariate analysis was undertaken using linear regression both unadjusted and fully adjusted for age, sex, and established hepatic risk factors.

A sensitivity analysis addressing missing CK18 and ELF data was performed using multiple imputation by chained equations(29). Data were considered to be missing completely at random as they were missing due to technical problems or insufficient stored sample.

Ethical approval was obtained from the Lothian Research Ethics Committee and all subjects gave written informed consent.

Results

Subject characteristics

Of the 939 ET2DS participants who underwent liver ultrasound and further physical and liver assessment, 899 did not have pre-diagnosed liver disease or CLD on screening and were considered for inclusion in the current analysis (15 subjects were excluded due to pre-diagnosed CLD, a further 3 due to CLD on screening and 22 because they did not have a liver screen when indicated). 825 and 568 of these subjects underwent measurement of CK18 and ELF respectively and form the primary study populations for this paper. A number of ELF measurements were missing due to inadequate sample volumes. Details of the flow of patients through the study are shown in figure 1 and characteristics of the study populations are described in table 1. Compared with all subjects undergoing ultrasound examination, the populations with CK18 and ELF data available had similar clinical and metabolic characteristics. Prevalence of steatosis was 56.8% and the prevalence of NAFL (defined as the presence of steatosis on ultrasound without alcohol excess, use of hepatotoxic medication or raised autoantibodies) 31.5%.

Biomarker distributions

CK18 values ranged from 29 to 993 U/L (median 102, IQR 76-137 U/L) and ELF scores ranged from 6.9 to 11.6 (mean 8.9, SD 0.8). Distributions of CK18 were similar in men and women (medians 104 vs 100 respectively, $p>0.05$) with ELF scores slightly lower in men (means 8.8, SD 0.7 and 9.0, SD 0.8, respectively, $p=0.016$). ELF, but not CK18, increased significantly with age ($r=0.28$, $p<0.001$ and $r=-0.08$, $p>0.05$ respectively) and the two markers significantly, though relatively weakly, correlated with each other ($r=0.13$, $p=0.002$).

Association of CK18 and ELF with hepatic steatosis and established hepatic risk factors

Subjects with hepatic steatosis (n=460) had higher CK18 values compared with those without steatosis (n=365) (medians 120.2 vs 87.7U/L respectively, $p<0.001$). The opposite was found for patients with NAFL (medians 87.4 vs 112.5 U/L, $p<0.001$). ELF scores were similar in participants with steatosis (n=319) compared with those without steatosis (n=259) (means 8.88 vs 8.90, $p>0.05$), with similar results for the presence of NAFL (means 8.95 vs 8.87, $p>0.05$).

The associations of CK18 with established hepatic risk factors (excess alcohol intake, positive immunology titres and hepatotoxic medication use) are shown in Table 2a, for all subjects (n=825) and for those with steatosis (n=460). After adjustment for age and sex, mean CK18 was significantly higher in subjects reporting excess alcohol intake but differences in positive immunology titres and hepatotoxic medication use were not statistically significant.

Similar findings for ELF are presented in table 2b, again for all subjects (n=568) and for those with steatosis (n=319). ELF appeared, if anything, slightly lower in subjects with established risk factors for liver dysfunction, but differences were not statistically significant.

Association of CK-18 and ELF with metabolic risk factors

Age and sex adjusted associations of CK18 and ELF with metabolic variables are shown in Table 2. Higher CK18 levels were significantly associated with hyperglycaemia, increased body fat (higher BMI and waist circumference) and with higher serum triglyceride levels. Only the association with waist circumference remained statistically significant when analyses were restricted to subjects with NAFL.

Higher ELF levels were significantly associated with increasing duration of diabetes, with hyperglycaemia and with increased body fat. Compared with subjects who were treated with diet alone, mean ELF was significantly higher in subjects using OAHA alone (means 8.9 vs 8.7, p=0.012) and in those using insulin (mean 9.2 vs 8.7, p<0.001). The associations with ELF changed little when analyses were restricted to only subjects with steatosis or NAFL.

Characteristics of subjects in highest CK-18 and ELF quintiles

Since particularly high levels of CK18 and ELF may be diagnostic of clinically important liver inflammation and fibrosis respectively, we determined the clinical characteristics of subjects in the top biomarker quintiles (Table 3).

Compared with subjects in the bottom four quintiles, subjects in the highest CK18 quintile had significantly higher indices of hyperglycaemia, higher triglyceride levels and increased body fat. In addition, more were on intensive diabetes treatment (including insulin) and more reported drinking excess alcohol. When analyses were restricted to subjects with NAFL, no statistically significant differences were found (data not shown).

Subjects in the highest ELF quintile were slightly older, had longer diabetes duration and were more likely to require insulin therapy. These statistically significant differences persisted when the analyses were restricted to subjects with NAFL (age: mean 69.4 vs 67.8 yrs, p=0.010; duration of diabetes: 6.97 vs 6.74 years, p=0.010, on insulin therapy: 27.5 vs 13.5%, p=0.021).

Subjects were also assessed for surrogate markers of advanced fibrosis. Subjects in the top ELF quintile had significantly higher mean spleen size (10.6 vs 10.1cm, p=0.020), APRI (0.34 vs 0.26, p<0.001), AST/ALT ratio (1.02 vs 0.93, p=0.011) and lower platelet count (246 vs 267 $x10^9$/L, p=0.005).

Liver biomarkers were not significantly associated with other conditions known to potentially affect their levels in the circulation. The top and lower CK18 quintiles had similar proportions of subjects with CKD (19.4% vs 18.3%, p=0.74) and the top and lower ELF quintiles had similar proportions of individuals with a diagnosis of arthritis (41.6% vs 38.0%, p>0.05).

Multivariate analysis

In multivariate models adjusting for age, sex and established hepatic risk factors (Table 4), statistically significant positive predictors of CK18 were presence of hepatic steatosis, serum triglycerides and measures of increased body fat, hyperglycaemia and more intensive diabetes treatment. Similar predictors for ELF were duration of diabetes, more intensive diabetes treatment and body fat. In these models, r^2 ranged from 2.6 to 10.1% for risk factors influencing CK18 and from 12.7 to 13.7% for risk factors influencing ELF.

Due to the relatively large number of subjects in whom ELF (and to a lesser extent, CK18) data were unavailable (i.e. 'missing' at random), a sensitivity analysis was undertaken using multiple imputation (imputation dataset included all 899 subjects eligible for inclusion in the analyses - see figure 1). The results confirmed those in the original dataset with only minimal differences found in effect sizes and significance levels (data available on request).

Discussion

The strength of this study is the comprehensive assessment of CK18 and ELF in a population-based cohort of all older people with type 2 diabetes, and not just subjects selected primarily on the diagnosis of steatosis of the liver using recruitment from diabetes clinics at tertiary referral centres as in previous studies(15, 30-32). We have provided diabetes-specific information on the distribution of these biomarkers, which is essential to inform further research on the clinical relevance of possible subclinical liver dysfunction in this high risk group. We also demonstrated that higher CK18 levels were associated with hepatic steatosis, excess alcohol intake, increased body fat, higher serum triglyceride and circulating glucose levels. ELF scores increased with age and duration of diabetes and were associated with increased body fat and more intensive diabetes treatment. A challenge in interpreting these results clinically is the lack of validated biomarker cut-points to diagnose hepatic inflammation and/or fibrosis in population-based cohorts. Despite this, our results suggest that at least a number of metabolic risk factors are likely to be associated with liver fibrosis and/or inflammation in people with type 2 diabetes.

Whilst accepted as imperfect, especially in NAFLD (8), liver biopsy remains the gold standard for staging liver disease, but biopsy is not acceptable in large studies of asymptomatic participants. Cut-points for the non-invasive biomarkers used in the current study have been well validated for staging liver disease in secondary care patient populations with an intrinsically higher prevalence of CLD, including NAFLD, but have not been validated as a diagnostic tool in either general population groups or in patients with type 2 diabetes. Given the considerable influence of disease prevalence on the predictive values of diagnostic tests, the results from hospital-based studies could not be transferred to our own community-based, 'low prevalence' population without resulting in an unacceptably high

number of false positive and negative results. In terms of diabetic populations, it is not known whether ELF scores differ, on average, from non-diabetic populations, although there is also no known biological reason why this should be the case. For these reasons, we chose the top quintile of the biomarker distribution as our highest risk groups. Whilst the imprecision of such an approach in terms of diagnosing disease must be acknowledged, we have shown that the highest ELF quintile contained higher surrogate markers of advanced fibrosis, providing some confirmatory evidence that, at least for ELF, this group included a particularly high risk group of patients in terms of advanced liver disease. In addition we were able to confirm that the presence of other conditions known to influence levels of the biomarkers do not appear to have a major effect on the results.

In terms of the distributions of potential inflammation and fibrosis biomarkers, our findings for CK18 were consistent with the assay literature(33). In developing the normal ranges for the serum CK18 assay, 200 'healthy' Swedish blood donors were tested; as in our study, the results showed similar levels in males and female with little change in levels with increasing age and an overall biomarker distribution similar to the one we found. In one study (33), a normal cut-point of the 80th percentile, or 145U/L, was suggested, and this is also consistent with our finding (146U/L). There is minimal literature examining ELF distributions in individuals unselected for liver disease. Yoo et al suggest a normal range of 5.95-8.73 in South Korean subjects without known CLD(14). We found that ELF scores were very slightly lower in men and increased with age. In the absence of a biologically plausible reason to expect any difference in any of the components of ELF by sex, it is possible that the higher ELF scores in females may truly represent more advanced liver disease. The components of ELF (HA, TIMP-1 and P3NP) are all related to extra-cellular matrix turnover and are not exclusive to the liver. As a result, one might expect an increase in ELF with age,

both due to the greater time in which liver fibrosis has had to develop(34) and due to increasing prevalence of unrelated causes of raised analytes and indeed, consistent with our own findings, an early study examining HA and P3NP found higher levels in 'healthy' elderly people compared with younger participants (35) .

Our finding of associations of hepatic steatosis, raised serum triglycerides and increased body fat, hyperglycaemia and more intensive diabetes treatment with CK18, and of duration of diabetes, more intensive diabetes treatment and body fat with ELF, support the possibility that poor diabetes control and a worse metabolic profile may be increasing the risk of developing CLD. Our findings contrast those of a recent liver biopsy study (15) in people with type 2 diabetes, in which high rates of both NASH (78%) and moderate fibrosis (34-60%) were detected, but which did not find associations between diabetes related/metabolic factors and NASH or liver fibrosis. However, this biopsy study was small (n=98) and focused on patients at the severe end of the diabetes spectrum attending a tertiary referral hospital.

The cross-sectional nature of our study limits any temporal inference; it is not possible to determine whether metabolic factors are a risk factor for liver disease or vice versa. However, if causal relationships were to be confirmed, this would have important implications for strategies aimed at CLD risk reduction e.g. losing/redistributing fat and reducing insulin resistance. Although associations between the biomarkers and metabolic factors appeared relatively weak, addressing even weak risk factors for disease could be beneficial at a population level, especially if those risk factors are highly prevalent.

In addition to the association of CK18 and ELF with metabolic risk factors, we were also interested in their association with steatosis and established hepatic risk factors. We found

that subjects with hepatic steatosis had higher CK18 levels, but not ELF, compared with non-steatotic individuals. This is perhaps unsurprising given that CK18 levels rise with increasing hepatic inflammation as a by-product of hepatocellular apoptosis and that according to established models of NAFLD progression(27, 36), initial development of hepatic steatosis is followed by NASH and then hepatic scarring with steatosis typically receding as fibrosis progresses. Conversely, patients fulfilling the criteria for NAFL had significantly lower levels. This suggests that the alternative causes of steatosis (hepatotoxic medications, alcohol and strongly positive autoantibody titres) are driving the inflammatory element, with those patients with NAFL having a more benign course. We found little evidence of a strong association between hepatic risk factors and either CK18 or ELF. Lack of associations may be explained, at least in part, by the small numbers of study participants with high levels of the hepatic risk factors and by lack of consensus around the precise level of risk factor which should be used to establish increased risk. We defined alcohol excess using cut-points which are consistent with the published literature in the UK, only six participants had positive autoantibodies and we were unable to find any consensus in the literature on how best to define hepatotoxic medications in terms of what types, duration and dosage are required to have a significant effect on the liver(2).

One of the most consistent findings in the current study was the association of both biomarkers with measures of increasing body fat. Previous studies have shown a direct association between liver fat and hepatic inflammation, with the latter increasing proportionately according to liver fat volume(37). It has been proposed that this effect is mediated though the direct release of toxic free fatty acid by hepatic fat and through altered lipid partitioning within hepatocytes, mitochondrial dysregulation, generation of reactive oxygen species, lipid peroxidation and endoplasmic reticulum stress(38). Given the

relationship between visceral fat and inflammation, our finding of increased body fat in patients in the highest CK18 quintile is consistent with proposed underlying mechanisms of hepatic inflammation(39, 40). In addition to increased body fat, subjects in the top CK18 quintile also had higher fasting glucose and plasma HbA1c levels, as well as more intensive diabetes treatment modalities. These factors may be considered as surrogates of beta cell failure and worsening insulin resistance, which is in turn related to hepatic inflammation through increased lipolysis, increased free fatty acid presence in the liver and ultimately oxidative stress(41). As in other studies (42, 43), we found higher triglyceride levels with increased CK18 levels, which would be consistent with the theory of free fatty acids driving lipid accumulation in the form of triglycerides in the liver in NAFL.

In conclusion, we have provided important new information on the distribution of CK18 and ELF in an elderly diabetic population unselected for liver disease. Also, we have provided evidence that CK18 and ELF are increased in those people with type 2 diabetes who have a more adverse metabolic profile, including higher levels of body fat, whilst established risk factors for CLD were not found to have a major influence of levels of the biomarkers. These findings could help identify particularly high risk groups within the diabetic population who may benefit from increased surveillance in relation to development of CLD and/or from targeting of specific metabolic risk factors. Prospective studies are now required to determine the extent to which CK18 and/or ELF predict the development of symptomatic liver disease and to identify additional risk factors responsible for the development of advanced liver disease in people with type 2 diabetes.

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Duality of interest

P.C.H. has spoken for Pfizer and received travel support from MSD, Janssen and Gilead. J.F.P. has received research support from Pfizer and Bayer Health Care Diagnostics Division. M.W.J.S. has received research support from Pfizer and funds to support staff members from Takeda UK and Sanofi-Aventis and was a consultant to and on the speakers' bureau for GlaxoSmithKline. No other potential conflicts of interest.

Contribution statement

J.R.M. designed the study, collected and analysed data and wrote the manuscript. J.A.F. researched data, contributed to the discussion and reviewed/edited the manuscript. R.M.W. collected data and reviewed/edited the manuscript. L.D.N. collected data and reviewed/edited the manuscript. A.P.J. collected data and reviewed/edited the manuscript. S.G. researched data and reviewed/edited the manuscript. R.M.R. researched data and reviewed/edited the manuscript. P.C.H. researched data, contributed to the discussion and reviewed/edited the manuscript. I.N.G researched data, contributed to the discussion and reviewed/edited the manuscript. M.W.J.S. designed the study, researched data, contributed to the discussion and reviewed/edited the manuscript. J.F.P. designed the study, researched and collected data, contributed to the discussion and reviewed/edited the manuscript.

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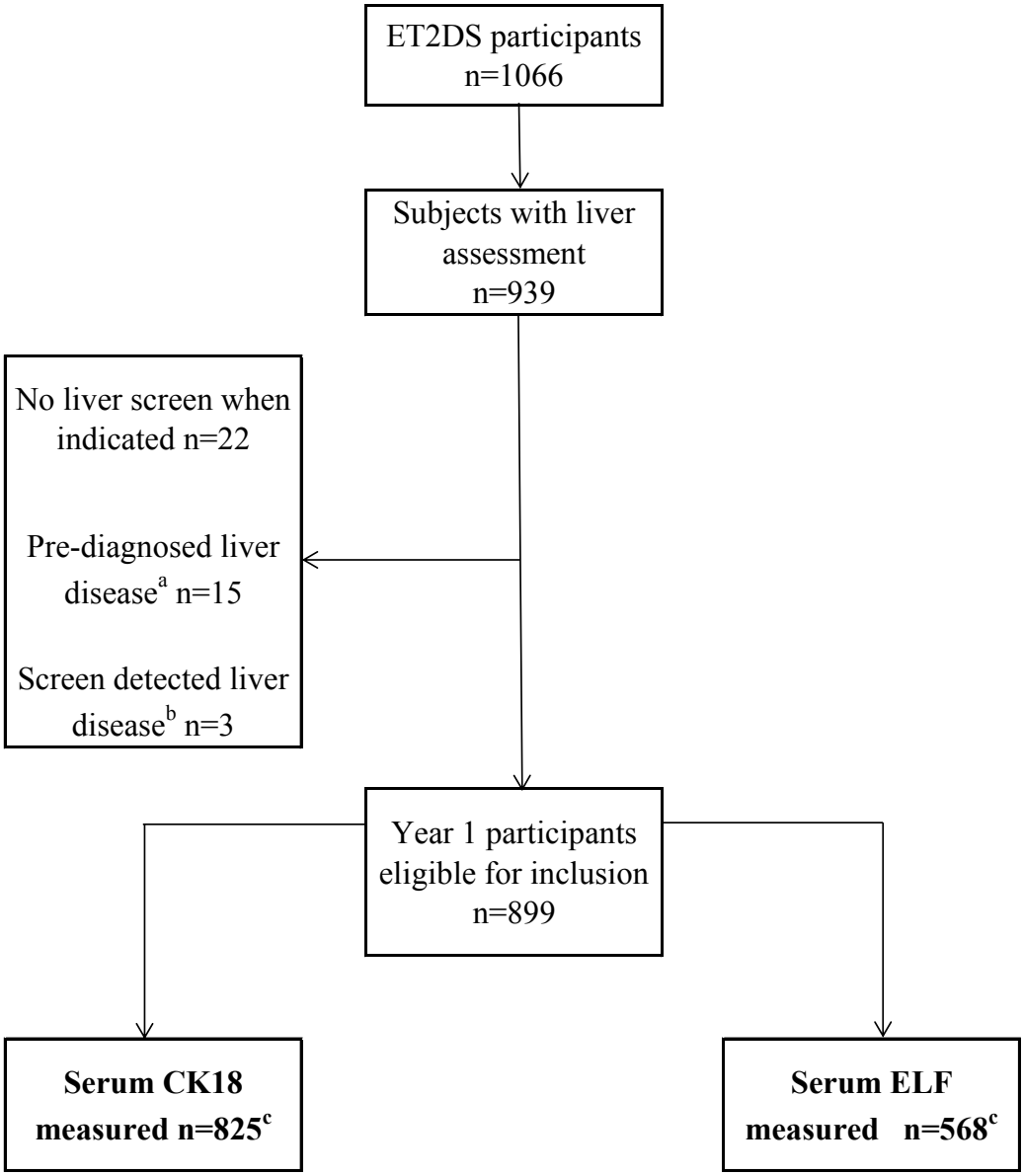
Figures and tables

Figure 1. Patient flow diagram



[a] Pre-diagnosed liver diseases (n=15) included alcohol related liver disease (n=7), autoimmune hepatitis (n=2), primary biliary cirrhosis (n=2), haemochromatosis (n=1), granulomatous hepatitis (n=1), chronic cholangitis (n=1) and carcinoid tumour (n=1).

[b] Screen-detected liver disease included hepatitis B virus n=1, hepatitis C virus n=1, hepatocellular carcinoma n=1

c Missing data values due to inadequate sample volumes.

Table 1. Characteristics[a] of all ET2DS participants undergoing liver assessment (n=939) and groups with CK18 (n=825) and ELF (n=568) measurements. Values are mean (SD)/median (IQR) or proportion (n)

	All participants n=939	CK18 participants n=825	ELF participants n=568
Age, years	68.9 (4.2)	68.8 (4.2)	68.7 (4.2)
Sex, % male	52.0 (488)	53.6 (442)	49.8 (283)
Duration of diabetes, years	7.0 (4.0-12.0)	7. (4.0-12.0)	7.0 (4.0-11.0)
HbA1c, %	7.19 (1.1)	7.19 (1.0)	7.20 (1.0)
HbA1c, mmol/mol	55.1 (11.7)	55.0 (11.4)	55.1 (12.3)
Fasting glucose, mmol/l	6.87 (2.3)	6.92 (2.3)	6.90 (2.3)
Diet controlled, % yes	19.4 (182)	19.8 (163)	18.8 (107)
OAHA use, % yes	64.9 (609)	65.3 (539)	66.7 (379)
Insulin therapy, % yes	15.8 (148)	14.9 (123)	14.4 (82)
BMI, kg/m^2	31.3 (5.7)	31.2 (5.6)	31.2 (5.7)
Waist circumference, cm	106.7 (12.8)	106.7 (12.7)	106.3 (12.5)
Serum total cholesterol, mmol/L	4.14 (0.8)	4.15 (0.8)	4.15 (0.8)
Systolic BP, mmHg	138.1 (18.5)	138.1 (18.2)	138.5 (18.1)
Diastolic BP, mmHg	74.1 (9.6)	74.4 (9.1)	74.6 (8.9)
Hepatic steatosis, % yes	56.8 (533)	55.8 (460)	56.2 (319)
NAFLD[b], % yes	31.5 (296)	32.4 (267)	31.5 (179)
Alcohol, units/week	1..3 (0-10.1)	2.3 (0-10.1)	0.6 (0-10.1)
Alcohol excess, % yes	12.2 (114)	11.8 (97)	12.4 (70)
Current smoker, % yes	13.0 (122)	13.1 (108)	12.7 (107)

[a] All variables were measured concurrently at year 1 examination of the ET2DS, except for BMI and waist circumference which were measured at baseline.

[b] Defined as the presence of steatosis on ultrasound without alcohol excess, use of hepatotoxic medication or raised autoantibodies

[c] Defined as females >14 units/week, males >21 units/week or patient disclosed history of a current or prior alcohol problem.

HbA1c, glycosylated haemoglobin; OAHA, oral anti-hyperglycaemic agent

Table 2a. Association of CK18 with metabolic and established hepatic risk factors. Values are age and sex adjusted correlation coefficients or mean (SEM)

		All patients with CK18[a] available n=825		Patients with steatosis n=460		Patients with NAFLD n=378	
Duration of diabetes[a], years		0.015	*NS*	0.043	*NS*	0.037	*NS*
Treatment type	*Diet controlled*	100.5 (1.8)		111.2 (3.0)		112.2 (3.4)	*NS*
	OAHA use[b]	109.7 (1.1)	*NS*	127.4 (1.8)	*NS*	123.3 (1.8)	*NS*
	Insulin therapy[b]	113.0 (2.4)	*NS*	136.8 (4.1)	*0.026*	126.2 (4.0)	*NS*
Metabolic risk factors							
Fasting glucose, mmol/L		0.093	*0.008*	0.112	*0.016*	0.062	*NS*
HbA1c, % and mmol/mol		0.064	*NS*	0.070	*NS*	0.048	*NS*
Body mass index, kg/m^2		0.117	*<0.001*	0.032	*NS*	0.085	*NS*
Waist circumference, cm		0.138	*<0.001*	0.061	*NS*	0.116	*0.024*
Serum total cholesterol, mmol/L		-0.051	*NS*	-0.041	*NS*	-0.073	*NS*
Serum triglycerides[a], mmol/L		0.142	*0.001*	0.064	*NS*	0.100	*NS*
Established hepatic risk factors							
Excess alcohol intake[c]	*Yes*	125.6 (3.0)		146.9 (4.7)			
	No	106.2 (1.0)	*0.004*	122.7 (1.5)	*0.002*		
Positive immunology[d]	*Yes*	120.5 (11.3)		97.5 (17.7)			
	No	110.2 (0.9)	*NS*	125.6 (1.4)	*NS*		
Hepatotoxic medication use[e]	*Yes*	121.1 (5.1)		143.2 (7.6)			
	No	107.9 (0.9)	*NS*	124.7 (1.5)	*NS*		

Table 2b. Association of ELF with markers of diabetes, the metabolic syndrome and liver dysfunction at year 1. Values are age and sex adjusted correlation coefficients or mean (SEM)

		All patients with ELF available n=568		Patients with steatosis n=319		Patients with NAFLD n=259	
Duration of diabetes[a], years		0.138	*0.001*	0.181	*0.001*	0.167	*0.008*
Treatment type	*Diet controlled*	8.71 (0.07)		8.68 (0.10)		8.73 (0.11)	
	OAHA use[a]	8.91 (0.04)	*0.012*	8.86 (0.05)	*NS*	8.89 (0.05)	*NS*
	Insulin therapy[a]	9.15 (0.08)	*<0.001*	9.21 (0.10)	*<0.001*	9.23 (0.11)	*0.002*
Metabolic risk factors							
Fasting glucose, mmol/L		0.048	*NS*	0.025	*NS*	-0.006	*NS*
HbA1c, % and mmol/mol		0.090	*0.033*	0.048	*NS*	0.074	*NS*
Body mass index, kg/m^2		0.170	*<0.001*	0.237	*<0.001*	0.252	*<0.001*
Waist circumference, cm		0.148	*<0.001*	0.175	*0.002*	0.192	*0.002*
Serum total cholesterol, mmol/L		-0.035	*NS*	-0.089	*NS*	-0.068	*NS*
Serum triglycerides*, mmol/L		-0.008	*NS*	-0.116	*NS*	-0.140	*NS*
Established hepatic risk factors							
Excess alcohol intake[b]	*Yes*	8.74 (0.09)		8.69 (0.11)			
	No	8.93 (0.03)	*NS*	8.91 (0.04)	*NS*		
Positive immunology[c]	*Yes*	8.61 (0.30)		8.31 (0.50)			
	No	8.90 (0.03)	*NS*	8.89 (0.04)	*NS*		
Hepatotoxic medication use[d]	*Yes*	8.85 (0.17)		8.77 (0.20)			
	No	8.91 (0.03)	*NS*	8.89 0.04)	*NS*		

[a] Analysed on the Log10 scale

[b] vs diet controlled

[c] Defined as females >14 units/week, males >21 unis/week or patient disclosed history of a current or prior alcohol problem.

[d] Defined as ASmA titer >1:160 or AMA titer >1:40

[e] Defined as the use of (non-topical) glucocorticoids for >1 week, isoniazid, methotrexate, amiodarone or tamoxifen within the 6 months prior to the year 1 clinic.

Continuous variables analysed using Pearson's correlation, categorical variables analysed using univariate analysis of variance.

CK18, cytokeratin-18; HbA1c, glycosylated haemoglobin; OAHA, oral anti-hyperglycaemic agent; SEM, standard error of the mean.

Table 3. Risk factors in highest versus lower quintiles of CK18 and ELF. Values are mean (SEM) or proportion (%)

	CK18 Quintile 1-4 n=660 (<146.6 U/L)	CK18 Quintile 5 n=165 (≥146.6 U/L)	p	ELF Quintile 1-4 n=455 (score <9.5)	ELF Quintile 5 n=113 (score ≥9.5)	p
Demographics						
Age	69.0 (0.16)	68.2 (4.6)	*NS*	68.3 (0.19)	70.6 (0.37)	*<0.001*
Sex, % male	54.5% (360)	49.7% (82)	*NS*	51.4% (234)	43.4% (49)	*NS*
Duration of diabetes[a], years	7.04 (0.08)	7.29 (0.16)	*NS*	6.75 (0.09)	7.93 (0.26)	*0.028*
Treatment type *Diet controlled*	22.0% (145)	10.9% (18)	*0.001*	20.2% (92)	13.3% (15)	*NS*
OAHA use	72.6% (479)	83.6% (138)	*0.004*	75.8% (307)	79.6% (90)	*NS*
Insulin therapy	13.9% (92)	18.8% (31)	*NS*	12.3% (56)	23.0% (26)	*0.007*
Metabolic risk factors						
Fasting glucose, mmol/L	6.80 (0.09)	7.36 (0.19)	*0.008*	6.86 (0.10)	7.07 (0.25)	*NS*
HbA1c, %	7.14 (0.04)	7.36 (0.08)	*0.013*	7.19 (0.05)	7.22 (0.10)	*NS*
HbA1c, mmol/mol	54.5 (0.44)	57.0 (0.91)		55.1 (0.53)	55.4 (1.09)	
Body mass index, kg/m^2	31.0 (0.22)	32.3 (0.45)	*0.009*	31.0 (0.26)	31.8 (0.55)	*NS*
Waist circumference, cm	106.2 (0.50)	108.7 (0.97)	*0.020*	105.9 (0.58)	107.7 (1.22)	*NS*
Total cholesterol, mmol/L	4.15 (0.04)	4.13 (0.07)	*NS*	4.17 (0.04)	4.09 (0.09)	*NS*
Triglycerides*, mmol/L	1.42 (0.01)	1.68 (0.03)	*0.001*	1.44 (0.02)	1.52 (0.03)	*NS*
USS detected hepatic steatosis						
Steatosis	49.4% (326)	81.2% (134)	*<0.001*	56.9% (259)	53.1% (60)	*NS*
Established hepatic risk factors						
Excess alcohol intake[b]	10.3% (68)	17.6% (29)	*0.014*	13.4% (61)	8.0% (9)	*NS*
Positive immunology titres[c]	0.7% (4)	1.2% (2)	*NS*	1.4% (6)	0% (0)	*NS*
Hepatotoxic medication use[d]	3.5% (23)	4.2% (7)	*NS*	3.5% (16)	2.7% (3)	*NS*

[a] Analysed on the Log10 scale

[b] Defined as females >14 units/week, males >21 unis/week or patient disclosed history of a current or prior alcohol problem.

[c] Defined as ASmA titer >1:160 or AMA titer >1:40

[d] Defined as the use of (non-topical) glucocorticoids for >1 week, isoniazid, methotrexate, amiodarone or tamoxifen within the 6 months prior to the year 1 clinic.

[e] Defined as eGFR<60 mls/min/1.73m^2

Continuous variables analysed using Students t-test, categorical variables analysed using Chi-square.

CKD Chronic kidney disease; CK18, cytokeratin-18; ELF, European Liver Fibrosis panel; HbA1c, glycosylated haemoglobin; OAHA, oral anti-hyperglycaemic agent; SEM, standard error of the mean.

Table 4. Multivariate association of risk factors with CK18 and ELF. Values are standardised beta coefficients (95% CI)

CK18	Model 1	p	R^2	Model 2	p	R^2
Hepatic steatosis	0.302 (0.23 to 0.37))	<0.001	0.090	0.292 (0.22 to 0.36)	<0.001	0.101
Triglycerides[a], mmol/L	0.148 (0.06 to 0.23)	<0.001	0.023	0.152 (0.07 to 0.23)	<0.001	0.050
Waist circumference, cm	0.142 (0.07 to 0.21)	<0.001	0.019	0.128 (0.06 to 0.20)	0.001	0.034
Fasting glucose, mmol/L	0.098 (0.03 to 0.17)	0.006	0.010	0.090 (0.02 to 0.16)	0.011	0.028
Body mass index, kg/m^2	0.100 (0.03 to 0.17)	0.006	0.010	0.098 (0.02 to 0.17)	0.009	0.027
Diet controlled	-0.085 (-0.16 to -0.02)	0.017	0.007	-0.091 (-0.16 to -0.02)	0.011	0.027
HbA1c, % or mmol/mol	0.086 (0.02 to 0.16)	0.034	0.007	0.081 (0.01 to 0.15)	0.025	0.026
Any OAHA use	0.079 (0.01 to 0.15)	0.029	0.006	0.085 (0.01 to 0.16)	0.018	0.026
Total cholesterol, mmol/L	-0.055 (-0.14 to 0.03)	NS	0.003	-0.062 (-0.15 to 0.02)	NS	0.028
Insulin therapy	0.030 (-0.04 to 0.10)	NS	0.001	0.028 (-0.05 to 0.10)	NS	0.019
Duration of diabetes[a], years	0.010 (-0.06 to 0.08)	NS	-	0.017 (-0.06 to 0.09)	NS	0.019

ELF	Model 1	p	R^2	Model 2	p	R^2
Body mass index, kg/m^2	0.154 (0.07 to 0.24)	<0.001	0.024	0.187 (0.10 to 0.27)	<0.001	0.137
Duration of diabetes[a], years	0.149 (0.06 to 0.23)	0.001	0.022	0.124 (0.04 to 0.21)	0.003	0.120
Insulin therapy	0.143 (0.06 to 0.23)	0.001	0.019	0.152 (0.07 to 0.24)	<0.001	0.127
Diet controlled	-0.110 (-0.20 to -0.02)	0.012	0.012	-0.116 (-0.20 to -0.03)	0.006	0.118
Waist circumference, cm	0.097 (0.01 to 0.19)	0.032	0.009	0.155 (0.07 to 0.24)	<0.001	0.126
Any OAHA use	0.059 (-0.03 to 0.15)	NS	0.003	0.064 (-0.20 to 0.15)	NS	0.109
HbA1c, % or mmol/mol	0.047 (-0.04 to 0.13)	NS	0.002	0.070 (-0.01 to 0.16)	NS	0.112
Hepatic steatosis	-0.029 (-0.12 to 0.06)	NS	0.001	0.014 (-0.07 to 0.10)	NS	0.105
Triglycerides[a], mmol/L	-0.025 (-0.13 to 0.08)	NS	0.001	-0.001 (-0.10 to 0.10)	NS	0.088
Fasting glucose, mmol/L	0.018 (-0.07 to 0.11)	NS	-	0.030 (-0.05 to 0.11)	NS	0.107
Total cholesterol, mmol/L	-0.003 (-0.11 to 0.10)	NS	-	-0.042 (-0.15 to 0.06)	NS	0.091

[a] Analysed on the Log10 scale

Model 1 - Unadjusted model, individual variables with no adjustment

Model 2 - Individual variables adjusted for age, sex, alcohol excess, hepatotoxic medication use and strongly positive autoantibodies

CK18, cytokeratin-18; ELF, European Liver Fibrosis panel; HbA1c, glycosylated haemoglobin; OAHA, oral anti-hyperglycaemic agent.